Pentose phosphate pathway

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2008

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Summary and discussion

The pentose phosphate pathway (PPP) provides an additional pathway for oxidation of glucose. In most tissues 80% to 90% of glucose oxidation is by glycolysis, and the remainder is oxidized by the PPP. In recent years, two defects in the PPP have been discovered [1-3]. Firstly, in 1999, our group reported on a patient with a slowly progressive leukoencephalopathy of unknown origin and massive accumulation of ribitol and D-arabitol in the brain and CSF and to a lesser extend in plasma and urine. In 2004, a deficiency of ribose-5-phosphate isomerase (RPI) was demonstrated in cultured cells from this patient and mutations were detected in the *RPI*a gene. Secondly, in 2001, the first patient with a deficiency of transaldolase (TALDO) was described. The disease was found in a teenage girl who had presented in the newborn period with an aortic coarctation, enlarged clitoris and mild bleeding tendencies. After several months she developed hepatosplenomegaly. In urine elevated concentrations of D-arabitol, ribitol and erythritol were found with only very mild elevations in plasma and CSF. Diagnosis was confirmed by the detection of a homozygous deletion of 3-bp in exon 5 of the *TALDO* gene and deficient TALDO activity in lymphoblasts at the age of ten. At that age she had developed liver cirrhosis and persistent hepatomegaly. A second patient with TALDO deficiency was detected in 2005 [4]. The girl was a newborn with severe liver failure and cardiomyopathy and she died at 18 days from respiratory failure.

The work presented in this thesis has mostly focused on improving the diagnosis of patients with a defect in the PPP, expanding the knowledge of these disorders and studying the function and importance of the PPP.

To improve the diagnosis of patients with a defect in the PPP and to investigate the intracellular concentrations of sugar-phosphates in these patients a new method was developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). With this method described in **chapter 2**, the accumulation of sedoheptulose-7-phosphate (sedoheptulose-7P) in blood spots, fibroblasts and lymphoblasts derived from TALDO deficient patients was established. In cells from the RPI deficient patient, no accumulation of sugar-P was detected suggesting an efficient conversion to pentoses and pentitols, which are strongly elevated in body fluids of the patient. Furthermore, the LC-MS/MS method can be used for determining enzyme activities of several steps of the PPP by measuring the sugar-P involved. This was done for TALDO and RPI deficiency.

Two methods were developed for the quantitative profiling of polyols in urine using LC-MS/MS (**chapter 3**). In the initial first-line screening method,

the different polyol isomers elute as one peak and cannot be distinguished from each other. A second highly selective method separates the different polyol isomers enabling the separate quantification of erythritol, threitol, ribitol, arabitol, xylitol, sorbitol, mannitol, galactitol, sedoheptitol and perseitol. Polyol profiles matching earlier described urinary concentrations of polyols for TALDO and RPI deficiencies and galactosemia were found. We were able to demonstrate for the first time accumulation of sedoheptitol and perseitol in patients with TALDO deficiency. These heptitols are thought to derive via a heptulose intermediate from sedoheptulose-7P. These methods can also be used for diagnosing other defects presenting with elevated polyol excretions and for monitoring galactitol excretion in patients with galactosemia after starting a galactose-restricted diet.

A LC-MS/MS method was established for identification and quantitation of the seven-carbon carbohydrates sedoheptulose and mannoheptulose in urine. Additionally, other seven-carbon chain carbohydrates including sedoheptitol, perseitol and sedoheptulose-7P were characterized in urine of four TALDO deficient patients (**chapter 4**). These patients had increased urinary concentrations of sedoheptulose and sedoheptulose-7P, associated with subtle elevations of mannoheptulose, sedoheptitol and perseitol. It was speculated that accumulated sedoheptulose-7P is most likely further metabolized to sedoheptulose, mannoheptulose, sedoheptitol and perseitol, perhaps as detoxification to lower the sugar-P levels which may have cytotoxic effects as has previously been suggested for galactose-1P in galactosemia [5,6]. The new LC-MS/MS allowed us to reveal novel urinary biomarkers for identification of TALDO deficiency.

The diagnosis of TALDO deficiency in a French family with four affected children born to the same consanguineous parents in 2006 expanded the number of TALDO deficient patients from two to six [7]. One of these patients presented in the antenatal period with hydrops foetalis with oligohydramnios. The pregnancy was medically terminated at 28 weeks gestation and amniotic fluid was saved. The newly developed methods were used to retrospectively measure in the amniotic fluid sample of this fetus, polyols, heptuloses and sedoheptulose-7P with LC-MS/MS (**chapter 5**). Our results demonstrated that TALDO deficiency results in elevated metabolite levels of sedoheptulose and ribitol in amniotic fluid, while erythritol, arabitol, mannoheptulose, sedoheptitol, perseitol and sedoheptulose-7P concentrations were normal. These findings show that the PPP is active during foetal development and that a defect of TALDO may cause alterations in early embryogenesis. Moreover this characteristic profile in the amniotic fluid may add, next to molecular investigations, to prenatal diagnosis for families with an index-case or for pregnancies complicated with early hydrops associated with oligoamnios.

As more patients are being diagnosed, the phenotype of TALDO deficiency becomes more characterized. In **chapter 6**, a new case of TALDO deficiency is described together with an overview of the clinical and biochemical findings of all known TALDO deficient patients. All patients were born to consanguineous parents and presented in the neonatal or antenatal period with hepatosplenomegaly, liver dysfunction, hepatic fibrosis and anemia. Most patients showed some dysmorphic features (e.g. anti-mongoloid slant, low set ears, cutis laxa), neonatal edema, congenital heart defects or renal problems. Mental and motor development was mostly normal. The most recently diagnosed patient had clinical symptoms as previously described in TALDO deficiency but is the first with rickets and deafness. Biochemical findings were similar to other TALDO deficient patients. The elevated urinary erythritol, ribitol, arabitol, sedoheptitol, perseitol, sedoheptulose, mannoheptulose and sedoheptulose-7P are all explained by the defect in the PPP. It is believed that sedoheptulose and sedoheptulose-7P accumulation in TALDO deficiency is the cause of the liver impairment and the fibrosis and that a TALDO deficiency results in decreased NADPH formation from the PPP resulting in low NADPH/NADP and NADH/NAD ratios. The altered cytosolic redox state might play a role in the pathogenesis in TALDO deficiency, some evidence can be found in the low concentrations of some metabolites in patients (i.e. cholesterol, estradiol, testosterone and 25-hydroxyvitamin D) that need NADPH for their formation.

Patients with cystinosis caused by the 57-kb deletion including the deletion of the *CTNS* (cystinosin) gene were found to excrete elevated concentrations of sedoheptulose in their urine. This 57-kb deletion also includes an adjacent gene *CARKL,* which encodes a protein that was predicted to function as a carbohydrate kinase. We showed that the CARKL protein is a sedoheptulokinase catalysing the reaction: Sedoheptulose + ATP → Sedoheptulose-7P + ADP (**chapter 7**). An enzyme assay for the phosphorylation of sedoheptulose was developed and decreased sedoheptulokinase activity in fibroblasts from patients with the 57-kb deletion was found. Fibroblasts from cystinosis patients with other mutations displayed normal enzyme activity. The possible function of sedoheptulokinase is to form sedoheptulose-7P from sedoheptulose when glyceraldehyde-3P is redirected from glycolysis to the PPP. Cystinosis patients with the common 57-kb deletion have the severe infantile nephropathic type and until now, no difference in the clinical phenotype between these patients and patients with other mutations causing the severe infantile nephropathic type has been found [8].

The main function of the oxidative part of the PPP is the production of NADPH from $NADP^+$ and thereby maintaining the cytoplasmic NADPH concentration. NADPH is important in the defense of oxidative stress caused by reactive oxygen species. The PPP is closely linked to glycolysis by the intermediates glyceraldehyde-3P en fructose-6P. In earlier studies it has been reported that enhanced activity of the PPP was observed in mammalian cells under oxidative stress. The exact mechanism of this has been unclear. Recently, it was discovered that yeast cells with reduced activity of the key glycolytic enzyme triosephosphate isomerase (TPI) exhibit an increased resistance to the thiol-oxidizing reagent diamide. We have shown that the underlying mechanism is based on a redirection of the metabolic flux from glycolysis to the PPP, increasing the redox state of the cytosolic NADP(H) pool (**chapter 8**). Remarkably, another key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is known to be inactivated in response to various oxidant treatments (e.g. H_2O_2), and our results show that this provokes a similar redirection of the metabolic flux. PPP intermediates (sugar-P and NADPH/$NADP^+$ ratio) were measured in the different cell lines and compared to the wild-type. The results clearly showed that a decrease in cellular TPI activity or inactivated GAPDH by H_2O_2 resulted in elevated levels of almost all the metabolites of the PPP and the NADPH/$NADP^+$ ratio. This effect is absent in yeast cells in which the first and rate-limiting step of the PPP is inhibited. To confirm the metabolic measurements a mathematical model was developed based on the known kinetic parameters (Km, Vmax) for the related enzymes. The simulations revealed that 13 of the 14 qualitative changes in metabolite concentrations were correctly predicted by the mathematical model. A difference between the experimental data and the predictions was only observed for the metabolite sedoheptulose-7P. At the time of these experiments the enzyme sedoheptulokinase (see chapter 7) was unknown and not used in the mathematical model, which might be the cause for this difference.
Our results imply that rerouting of the metabolic flux is a basic mechanism in counteracting oxidative stress that is naturally switched on in the course of GAPDH inactivation. Patients with a defect in the PPP or glycolysis might have difficulties in reacting appropriately during oxidative stress. Yeast knock-out models can be used to show the biochemical response to oxidative stress compared to wild-type.

Finally, **chapter 9** summarizes the current knowledge of the metabolic routes (PPP, glycolysis, glucuronic acid pathway) involving sugars, polyols and sugar-P metabolism, including the clinical description of patients with a defect in the PPP and how they can be diagnosed using biochemical and molecular techniques.

Comparison of the clinical phenotypes of patients with a defect in the oxidative part of the PPP clearly shows that RPI deficiency results in severe brain abnormalities whereas in TALDO deficiency the most pronounced feature is liver cirrhosis. Interestingly, the clinical phenotype of patients affected with classical galactosemia, a disorder of galactose metabolism, is a combination of both brain and liver abnormalities with cataract as an additional feature. Classical galactosemia is biochemically characterized by the accumulation of galactose and galactose-1P and the polyol galactitol. There is evidence that the accumulation of galactitol is related to the brain and eye involvement, whereas the hepatic dysfunction is ascribed to the toxicity of galactose-1P [5,6]. In diabetes mellitus, there is evidence of neurotoxicity induced by another polyol i.e. sorbitol [9]. The above mentioned examples imply that supra physiological levels of polyols in the CNS (RPI deficiency) can be causative for brain damage, while intracellular increases of sugar-P (TALDO deficiency) results in internal organ damage with the liver being most severely affected. The functions of these two enzymes in the brain have not been thoroughly investigated. In the RPI deficient patient there is a high brain: CSF: plasma gradient of ribitol and D-arabitol. This finding suggest that RPI is normally of great importance in the brain while absence of neurological problems and normal brain and CSF polyol levels in TALDO deficiency suggest that TALDO is of minor importance in brain or that the brain has alternative pathways by-passing the metabolic block.
In both RPI and TALDO deficiency, a decreased NADPH production through the oxidative part of the PPP is expected to be involved in the pathogenesis of these disorders. NADPH is used in many anabolic pathways, such as lipid synthesis, cholesterol synthesis and fatty acid chain elongation. Lipids and cholesterol are both components of myelin synthesis, which is clearly abnormal in RPI deficiency. In patients with TALDO deficiency, there appears to be decreased activity of some of the NADPH-dependent reactions involving hormone, cholesterol and 25-hydroxyvitamin D synthesis. Hemolytic anemia was present in most patients (chapter 6), which might be caused by decreased NADPH production in erythrocytes.

Both RPI and TALDO deficiencies are likely to be under-diagnosed since both diseases have recently been described and are rather unknown, and the limited availability of specific analytical techniques which are required for the measurements of the appropriate biomarkers. We feel it is legitimate to screen all patients with unexplained hepatosplenomegaly, liver function problems, hepatic fibrosis and hemolytic anemia presenting in the neonatal or antenatal period and patients with neonatal hemochromatosis for TALDO deficiency by measuring urinary polyols and/or seven-carbon sugars. In addition, all patients with a poorly understood neurodegenerative clinical

course including leukoencephalopathy should be screened for RPI deficiency by measuring urinary polyols.

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